The *Arabidopsis* AtEPR1 extensin-like gene is specifically expressed in endosperm during seed germination

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Summary

Screening of 10000 *Arabidopsis* transgenic lines carrying a gene-trap (GUS) construct has been undertaken to identify markers of seed germination. One of these lines showed GUS activity restricted to the endosperm, at the micropylar end of the germinating seed. The genomic DNA 5’-anking the T-DNA insert was cloned by walking PCR and the insertion was shown to be located 70 bp upstream of a 2285 bp open reading frame (AtEPR1) sharing strong similarities with extensins. The AtEPR1 open reading frame consists of 40 proline-rich repeats and is expressed in both wild-type and mutant lines. The expression of the AtEPR1 gene appears to be under positive control of gibberellic acid, but is not downregulated by abscisic acid during seed germination. No expression was detected in organs other than endosperm during seed germination. The putative role of AtEPR1 is discussed in the light of its specific expression in relation to seed germination.

Keywords: gibberellins, endosperm, extensin, germination.

Introduction

In many plant species, after maturation and desiccation seeds are said to be dormant – unable to germinate under permissive conditions – for a period that depends on their genotype and environmental factors. Once primary dormancy is lost, usually after a dry storage period (after ripening), and if placed under favourable environmental conditions, a seed is capable of undergoing germination. Germination requires a group of complex physiological and developmental processes including rehydration, repair mechanisms (e.g. for DNA, proteins and membranes), triggering of metabolism, cell elongation, etc., leading to root-tip protrusion through the endosperm and testa.

Dormancy and germination represent fundamental steps in the plant’s life cycle and are important in the elaboration of crop yield. The importance of plant hormones, specifically abscisic acid (ABA) and gibberellins (GAs), which respectively inhibit and promote germination, has been known for a long time (Hilhorst and Karssen, 1992; Karssen et al., 1983; Koornneef and Van der Veen, 1980). The role of light, and more specially of the phytochromes, is also well documented (Reed et al., 1994; Shinomura et al., 1994) and mutants affected in light-signalling transduction, GA/ABA biosynthesis or sensitivity have been identified. More recently, new mutants with reduced dormancy (rdo) have been isolated. At least one does not appear to be affected in its ABA sensitivity/biosynthesis (Leon-Kloosterziel, 1996). Although genetic analyses in crop species as well as in *Arabidopsis* have allowed the identification of quantitative trait loci controlling dormancy (for review see Li and Foley, 1997), the genetic analysis of other factors involved in germination remains limited, possibly due to the occurrence of gene redundancy, or due to their involvement in other processes essential for plant viability.

Several genes have been found to be expressed at the onset of germination, their expression being spatially regulated during seed germination or post-germinative development. Nevertheless, most are also expressed during embryogenesis (Harada et al., 1988). More recently, proteins and mRNA that are differentially accumulated in dormant versus non-dormant seeds have been identified (Dyer, 1993; Johnson et al., 1995). In most cases the
function of these genes controlling seed germination is still speculative. As outlined by Bewley (1997), data on molecular mechanisms involved in both dormancy breakage and germination are scarce, and more molecular tools are required to improve our knowledge.

Gene trapping is a powerful approach that can be applied to plant development (for review see Topping and Lindsey, 1995). It is based on the transcriptional or translational fusion of a reporter gene with an endogenous gene. The expression of the transgene thus reflects the expression of the endogenous gene. Different systems have been widely used (Sundaresan et al., 1995; Vroemen et al., 1998) and have proved their efficiency for the analysis of complex developmental processes. Furthermore, gene trapping is a good alternative to overcome the problems of structural and functional redundancy encountered with genetic approaches. Using this approach, we have identified a number of molecular markers that are temporally or spatially regulated during imbibition.

Here we describe a transgenic T-DNA insertion line (DFJ48) expressing a GUS reporter gene specifically in the endosperm during seed germination. The genomic DNA corresponding to the insertion locus was cloned, and a putative gene identified as a new extensin gene. The role of this protein during seed germination is discussed in the light of its specific expression pattern.

Results

Isolation of the DFJ48 line

Screening of a collection of T-DNA lines was undertaken on the basis of GUS expression in imbibed seeds. This led to the isolation of 20 lines. DFJ48, exhibiting GUS activity in the endosperm specifically at the micropylar end of the germinating seed, was further characterized (Figure 1a). During seed imbibition, expression of the GUS reporter gene was found in the region of radicle protrusion, 30 h after the start of imbibition. During root protrusion through the testa no GUS expression was found in the root itself, but only in the tissues surrounding it (Figure 1a). Sections were performed on germinating seeds after GUS staining in order to discriminate between seed coat expression, endosperm and zygotic expression of the GUS reporter gene. Expression was detected specifically in the endosperm, as shown in Figure 1(c,d), but not in other tissues of the seed. As the plantlet grew, expression was not detected in any other organs such as meristems, leaves or stems, or in pollen or flowers (Figure 1b). In the case of GUS expression in the inner testa layer, a tissue of maternal origin, one would expect a maternal inheritance of the GUS expression. But this was found not to be the case, in agreement with the endosperm localization of GUS expression.

<table>
<thead>
<tr>
<th>[DFJ48 x DFJ48] generation</th>
<th>Number of seeds in progeny</th>
<th>Kan&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Kan&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Total</th>
<th>χ&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4</td>
<td></td>
<td>669</td>
<td>211</td>
<td>880</td>
<td>0.18</td>
</tr>
<tr>
<td>Obtained</td>
<td></td>
<td>660</td>
<td>220</td>
<td>880</td>
<td></td>
</tr>
<tr>
<td>Theoretical</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td></td>
<td>593</td>
<td>213</td>
<td>806</td>
<td>0.328</td>
</tr>
<tr>
<td>Obtained</td>
<td></td>
<td>604.5</td>
<td>201.4</td>
<td>806</td>
<td></td>
</tr>
<tr>
<td>Theoretical</td>
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</table>

The progenies of nine T3 hemizygous plants were analysed for segregation of the kanamycin resistance marker 14 days after sowing on a kanamycin-containing medium. Hemizygous plants were selected for each of the lines and tested in their progeny (T5). The one-quarter Kan<sup>2</sup>—three quarters Kan<sup>1</sup> segregations can be accepted with a risk of 5%.

Characterization of the insertion

A genetic analysis of the insertion locus was performed on heterozygous plants for the markers. The GUS reporter gene was transmitted as a typical dominant Mendelian marker in the progeny of line DFJ48 (data not shown). A co-segregation between the kanamycin and the GUS markers was always observed indicating one single T-DNA locus being responsible for both markers (Table 1).

Molecular identification of the insertion site

The genomic border at the GUS end of the T-DNA was cloned by walking PCR (Devic et al., 1997). Three different amplification products (850, 750 and 600 bp) were obtained after restriction with three different enzymes and directly sequenced using primers corresponding to the residual T-DNA. The three sequences matched a BAC sequence (AC006233), originating from the Arabidopsis genome-project, for chromosome 2, between bases 75327 and 74739 (Figure 2). No similarity with known genes was found at the nucleic acid level, but a 2285 bp open reading frame (ORF) was predicted 72 bp downstream of the insertion locus. To confirm that the DFJ48 line has an insertion corresponding to the sequenced border, primers were designed according to the genomic sequence surrounding the insertion locus, and a PCR-based analysis of the locus was performed with both genomic and GUS-specific primers, as presented in Figure 3. The T-DNA insertion corresponded to the location predicted by the amplified T-DNA border, which has been sequenced. The sequence of the PCR fragment obtained indicated that a 38 bp deletion occurred in the DFJ48 line, between ~34 and ~72 bp upstream of the AtEPR1 ATG putative start codon, at the GUS end of the insertion site.
Extensin expression during germination

Figure 1. GUS expression pattern in the DFJ48 line.
GUS activity was monitored at different stages during seedling development.
(a) Time course of expression during seed germination (24-36-48-55-60-69-72 h after imbibition start).
(b) 7-day-old plant; no expression was detected except for the residual seed endosperm close to the hypocotyl.
(c,d) Transversal and longitudinal sections, respectively, through the germinating seed: the endosperm is one cell-layer thick and shows GUS expression in the cells surrounding the protrusion of the radicle.
(e) Expression pattern of the GUS reporter gene in an embryo defective line mutant unable to germinate: expression is observed in all the endosperm.
(f) Expression pattern of the GUS reporter gene in a GA-deficient line (ga1-3). No expression is detected in the absence of GA3 (10^{-6} M) application during seed imbibition.
(g) GUS expression pattern in the progeny of primary transformants for the AtEPR1 promoter::GUS construct.
end, endosperm; rt, root tip; cot, cotyledon; hyp, hypocotyl. a,b,e,g,f, bar = 1 mm; c,d, bar = 0.1 mm.

Figure 2. Site of T-DNA insertion in the DFJ48 line. The genomic DNA flanking the T-DNA border of the DFJ48 line was cloned and used for sequence comparison in the databases. The cloned sequence was assigned to a genomic BAC sequence (AC006233). The presence of a putative open reading frame is given as a hatched box and the T-DNA insertion site is shown.

Figure 3. Verification of the structure of the insertion locus in the DFJ48 line.
Primer were designed on the genomic sequence at the vicinity of the putative T-DNA insertion locus 5’p, 5’-GATCGAGTTGGAGAAAGC-3’ and AtEPR1 3’, 5’-ATGGATGTTGGAGCGTTTGC-3’), and a PCR amplification was performed with one of these primers and a T-DNA specific primer (tag1, 5’-GGAGCTACATAGAAGGACTG-3’). No amplification was obtained from lines homozygous for the T-DNA in DFJ48 plants due to T-DNA length with primers 5’p and AtEPR1 3’, whereas amplification products of the right size were obtained with primers 5’p and tag1, indicating the T-DNA has inserted at the presumed locus. WT, wild-type control plant.

Furthermore, a 14 bp fragment sharing no similarity in the databases was found between the right T-DNA border and the genomic AtEPR1 sequence. This strongly suggests that GUS expression is driven by the promoter of this ORF. 

Structure of the AtEPR1 gene
The ORF downstream of the insertion is 2285 bp in length, beginning with a consensus start codon and finishing with a stop codon. The inserted T-DNA does not disrupt the putative coding sequence. No splicing site was identified using annotation programs (http://CCG-081.mit.edu/GENSCAN.html), suggesting that no intron is present in this putative gene. Arabidopsis genomic DNA was digested with several restriction enzymes, blotted onto membrane and hybridized to an 855 amplification product of the ORF. A single hybridization product was detected for each restriction enzyme (not shown), in agreement with the length predicted from the BAC sequence. This indicates that the AtEPR1 gene is a single-copy gene in Arabidopsis.

The deduced amino-acid sequence consists of 762 amino acids, with a predicted molecular mass of 81,894 Da. The polypeptide contains two putative domains. The first is a putative transmembrane helix domain of 17 amino-acid residues at the N terminus; the second domain is 655 amino-acids long and composed of 40 repeats of 17 amino-acid residues (Figure 4b) with a high content of proline residues (43.7% of the total amino-acid content). The two domains are separated by a proline-rich region of 28 amino-acid residues which does not share any similarity with the repeats.

The repeat unit is made of YSPxYPPP x1-x2-x3-PPTPT where x1 can be Ile or Val; x2 Gln, His or Lys; and x3 Lys, Met or Pro. The highly organized repeats are hydrophilic, as shown on the Kyte and Doolittle plot (Figure 4a), and the N-terminal region of the putative protein consists of a lipophilic tail of 40 amino-acid residues. The entire AtEPR1 protein is hydrophilic except for the N-terminal domain. The putative polypeptide of AtEPR1 consists of Pro (43.7%), Thr (9.8%), Lys (9.2%), Val (7%), Tyr (6.7%), Ser (6.4%), Ile (6.4%) Gln (2.8%), His (2.8%), Leu (1.3%), Ala (0.9%), Gly (0.8%), Asp (0.5%), Phe (0.4%), Asn (0.4%), Arg (0.4%), Met (0.3%) and Glu (0.1%). Pro represents 43.7% of the total amino-acid content, and Thr, Lys, Val, Tyr, Ser and Ile, 45.5% altogether. This type of structure is typical of extensins, a class of cell-wall structural protein.

Similarities to known extensins
A phylogenetic tree was constructed using the parsimony method of the PAsP software (Swofford, 1991) as shown in Figure 5. The amino-acid sequence of the putative protein was compared to 22 dicot and three monocot extensins, from the GenBank database. The analysis was conducted on the same sample of sequences used by Ahn et al. (1996)
in order to position AtEPR1 among plant proline-rich proteins.

Expression of the AtEPR1 gene in the wild type

To examine whether the ORF is transcribed, the expression of the putative coding sequence of AtEPR1 was monitored by RT–PCR analyses, as direct transcript detection by Northern blot analysis was below the detection limit under our conditions. This is not surprising as GUS activity suggests that the gene is specifically expressed in one cell layer (the endosperm) during germination. Oligonucleotides were designed at the 5’ end (AtEPR1 5’: CTTAGTGGTGCTTCAGTTGC) and 855 bp downstream (AtEPR1 3’: ATTGGTGTTGAG-GCGTTTGC) ends of the ORF. The results presented in Figure 6 show that a transcript can be detected in wild-type germinating seeds, indicating that this ORF does correspond to a transcriptionally active gene. Furthermore, an Arabidopsis expressed sequence tag (AI99324) sharing 99% identity, at the nucleic acid level, with the 3’ region of the ORF was found in the databases, in agreement with the transcriptional activity of the AtEPR1 gene. No expression was found in other organs or later during plant development. This demonstrates that the GUS expression pattern is similar to the genuine AtEPR1 expression. The same experiment was performed on mRNAs extracted from germinating DFJ48 seeds and a signal was detected, suggesting that its transcription is still active in the insertion line.
Figure 5. Phylogenetic status of AtEPR1 among extensins. Amino-acid sequences of 25 plant extensins were obtained from the Genbank database and were compared using three monocot and one animal (Caenorhabditis elegans) extensin as references. Cowpea extensin (X98628), soybean extensin (L22029), tomato extensin (M76870), tomato-2 extensin (X56865), soybean-2 extensin (L20303), cowpea-2 extensin (X86030), bean extensin (M18093), bean-2 extensin (M18095), cowpea-3 extensin (X86030), tomato-3 extensin (X56881), carrot extensin (X027873), potato extensin (Z21937), Nicotiana plumbaginifolia extensin (M34371), tobacco-1 extensin (D13951), tobacco-2 extensin (X71602), sunflower extensin (M76546), rape extensin (A19812), Arabidopsis extensin (Z18787), tobacco-3 extensin (X13885), tomato-4 extensin (M76617), Arabidopsis extensin (Atext1), sorghum extensin (X56010), rice extensin (X61280), Zea mays extensin (X63134).

Hormonal control of GUS expression

To test such control of AtEPR1 expression, an analysis of GUS activity was performed after addition of hormones or inhibitors of their biosynthesis. The results are presented in Table 2. GUS expression was not repressed by ABA application, at a concentration of 10^-4 M, which inhibited protrusion of the radicle (Koornneef et al., 1982). No expression was detected in paclobutrazol-treated seeds at a concentration of 10^-4 M, which also prevented germination by inhibiting gibberellin biosynthesis (Rademacher and Graebe, 1979). Both germination and expression were restored by combined paclobutrazol and GA3 application. Methyl jasmonate or salicylic acid, which have been shown to play a role in the regulation of another extensin (Merkouropoulos et al., 1999), did not modify the GUS expression pattern or germination behaviour under our conditions.

The DFJ48 line was crossed with a GA-deficient line (ga1-3 NW58) to confirm the control of AtEPR1 expression by GAs. F2 lines were obtained with the DFJ48 insertion locus in a GA-deficient background. Seeds were monitored for GUS activity with and without applications of GA3. The results, presented in Figure 1(f), show that no expression or germination was obtained without GA treatment, confirming the positive role of GAs in the control of AtEPR1 expression.

Other controls of GUS expression

As the elongating root in the germinating seed applies pressure to the endosperm, the hypothesis was examined that AtEPR1 expression could be related to the pressure applied by the radicle protrusion. The DFJ48 line was crossed with a T-DNA mutant line defective in embryo development (emb). This line has been characterized as exhibiting developmental arrest during late embryogenesis, preventing homozygous seeds from germinating (M. Boisson, unpublished results). This emb mutant does not germinate, although a seed swelling can be observed during rehydration leading to tearing of the testa. Heterozygous emb and homozygous DFJ48 were crossed and F2 seeds were selected based on the lack of seed germination. The non-germinating seeds, obtained at the expected ratio, were monitored for GUS activity, and results are presented in Figure 1(e). The expression of the GUS reporter gene was also detected in this non-germinating emb background. The pattern found in this

Figure 6. RT-PCR detection of the putative RNA for AtEPR1 messenger. (a) AtEPR1 messenger RNAs were reverse transcribed and PCR amplified with primers located at the 5' end of the coding sequence (AtEPR1 5', 5'-GATGAGTGTCGAGAAACG-3') and 855 bp downstream (AtEPR1 3', 5'-TTGGATCTCTTGAGAAATATG-3'). The putative gene encoding a proline-rich protein is transcribed in the wild-type plants. As a control, primers EF1a4 up-low are amplifying an 814 bp genomic DNA fragment containing a 107 bp intron (Alexel et al., 1989). (b) RT-PCR detection of the AtEPR1 cDNA in a DFJ48 background compared to the control. The 855 bp amplification product is found in DFJ48, indicating a transcription of this gene in the mutant.
Table 2. Hormonal control of GUS expression

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GUS activity</th>
<th>Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Paclobutrazol</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ABA/GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Paclo/GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl jasmonate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Water</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Various treatments known to inhibit (ABA, paclobutrazol) or promote (GA<sub>3</sub>) seed germination were performed on seed germinated in the light. Both GUS activity and completion of seed germination were monitored. No expression was obtained under paclobutrazol treatments and no germination occurred. However, ABA was able to prevent seed germination without inhibiting GUS activity. ABA, paclobutrazol, GA<sub>3</sub>, salicylic acid, methyl jasmonate all 10<sup>-4</sup>M.

background was not strictly related to the micropylar end of the germinating seed, but was present in the whole endosperm. This demonstrates that expression of *AtEPR1* is not directly related to radicle protrusion, but may be promoted by the pressure exerted on the endosperm by swelling or due to other aspects of seed germination.

**Functional analysis of AtEPR1 promoter**

To identify a genomic region sufficient to confer this specific regulation, an 855 bp fragment flanking the T-DNA insert upstream of the GUS reporter gene was tested by transformation. This sequence was cloned into a blunt-ended pMECA plasmid and subcloned into a binary vector carrying a promoterless GUS reporter gene (pB1101, Clontech, Palo Alto, CA, USA). This plasmid was transferred to agrobacteria and vacuum-infiltrated into Ws Arabidopsis plants, and the progeny of the transformants obtained were tested for GUS activity during seed germination. Several primary transformants were obtained and revealed specific GUS expression during seed germination in the endosperm (Figure 1g), demonstrating the functionality and specificity of the cloned promoter. No GUS expression was observed in response to wounding of leaves of the transformants obtained.

**Discussion**

In this paper we describe the isolation and characterization of the AtEPR1 gene that encodes a protein with a domain having repeated motifs similar to extensins. AtEPR1 is specifically expressed in the endosperm during seed germination, under the control of GAs. Its promoter (approximately 800 bp) is sufficient to confer this specific regulation to a reporter gene.

**Identification of the AtEPR1 gene**

The use of a specific pooling strategy (see Experimental procedures) allows large-scale screens among a T-DNA collection, at a very low time cost, provided that the screening depends on a positive selection. The method applied in this study should allow faster identification of a line of specific interest, and easier manipulation of scoring data. The cloned genomic border of the T-DNA allowed identification of the insertion locus upstream of a gene containing proline-rich repeated sequences. The repetitive motif found in the AtEPR1 gene appears to share strong similarities with extensin proteins, although it does not exactly match the canonical Ser-Pro<sub>4</sub> motifs. The amino-acid comparison of AtEPR1 sequence to known extensins showed that three major groups of proteins emerged on the phylogenetic tree (Figure 5). Group 1 contains extensins with a highly conserved Pro-Val-Tyr-Lys-Ser-(Pro)4 protein motif, whereas group 2 extensins are more Tyr-rich with a Tyr-Tyr/Val-Tyr-Lys-Ser-(Pro) domain. Group 3 contains both monocot and dicot extensins, including AtEPR1, bean (m18093) and tomato (x55686) extensins that are closely related. Interestingly, the fact that AtEPR1 is more closely related to several dicot and monocot proteins than to two other Arabidopsis extensins (group 1) suggests that at least two types of extensin were present in the common ancestor of both monocots and dicots.

**Regulation of AtEPR1 expression**

The AtEPR1 gene is specifically expressed in the endosperm during seed germination, at the site of radicle protrusion. AtEPR1 expression requires GAs as demonstrated by the absence of GUS activity in both GA-deficient background and in wild-type seeds treated with a GA biosynthetic inhibitor. ABA treatments prevent seed germination but do not block AtEPR1-driven GUS expression. This indicates that inhibition of germination by ABA probably occurs late in the cascade of events leading to germination, in agreement with the hormonal regulation of the expression of several other markers (Dubreucq et al., 2000). AtEPR1 expression is not induced by wounding, and no modification of the expression pattern was obtained after treatments with either salicylic acid or methyl jasmonate, signalling biotic as well as abiotic stresses. However, the expression may be induced by mechanical constraints applied by the expanding root on the endosperm, as shown by analyses of the double mutant with a germination-deficient line. If AtEPR1 is expressed during seed imbibition in response to the mechanical stress induced by cell expansion, one may expect that a mutation affecting the expression of this gene will not affect germination behaviour.

Lack of phenotype of the insertion mutant

On this line, no phenotype associated with the segregation of the T-DNA was observed under our conditions. Germination speed and germination rate do not show any difference between the DFJ48 homozygous line and the wild-type (data not shown). However, AtEPR1 mRNA was detected in the homozygous DFJ48 line, despite a 7 kb insertion 72 bp upstream of the start codon, providing a possible explanation for the lack of phenotype. It is possible that the 3S promoter, which is driving the expression of the Basta resistance gene in the inserted T-DNA, has an effect on the transcription of the AtEPR1-coding sequence downstream of the insertion, and that a transcription product is subsequently found in the homozygous mutant. This has already been observed in other T-DNA lines (D. Bouchez, personal communication). Since the DFJ48 mutant is not a loss-of-function mutant, it is not yet possible to draw firm conclusions from the lack of phenotype of this line.

Is AtEPR1 involved in seed germination?

Several hydrolytic enzymes, such as endo β-mannanase or xylanase, implicated in cell-wall modifications, are known to be activated during seed imbibition (Dahal et al., 1997; Still and Bradford, 1997; Voigt and Bewley, 1996). Some of these enzymes are expressed specifically in the endosperm (Groot and Karssen, 1987; Toorop et al., 1996), such as endo-β-1-3 glucanase (Leubner-Metzger et al., 1995); β1-4 mannan endohydrolase (Bewley, 1997); and cellulases (Sanchez et al., 1986). They may act on the weakening of the endosperm at the seed micropylar end, where the radicle is protruding (Groot and Karssen, 1987). The role of GAs in promoting seed germination and of ABA in preventing it is well established (koorrieneef et al., 1982). A first link with hydrolytic activity was established when it was shown that the puncture force of the tomato endosperm cap does not decrease in the presence of ABA (Groot and Karssen, 1992). An endo-β-mannanase was shown to be downregulated by ABA and upregulated by GAs (Nomaguchi et al., 1995), which also correlates the antagonism effects of ABA and GAs on germination. However, despite their interesting localization, the activity of such enzymes may not be strictly related to seed germination (Toorop et al., 1996).

Recently, specific extensins have been demonstrated to be expressed in elongating cells and may have an important role in cell-wall structure (Bernhardt and Tierney, 2000). Furthermore, a modifying role has already been suggested for some non-standard proline-rich proteins (Keller and Lamb, 1989). The authors have found a new hydroxyproline-rich glycoprotein expressed specifically in a set of cells involved in the initiation of the lateral root. The authors suggested that the activation of genes encoding specific structural proteins would provide a mechanism for morphogenetic control of cell-wall architecture during cellular differentiation. Based on previously described data, as well as the spatial and temporal regulation of the AtEPR1 gene expression, we may hypothesize a more specific role of the protein in modifying the cell-wall structure, specifically during seed germination, thus facilitating radicle protrusion. Since the endosperm in Arabidopsis consists, at this stage, of a single cell layer (Berger, 1999), it is possible that cell-wall modifications may primarily modify the structure and physical properties of the dried tissues of the testa, which was shown to limit the germination rate. For instance, Arabidopsis transparent testa (tt) or aberrant testa shape (ats) mutants, which have a modified testa structure, have been shown to exhibit faster germination (Debeaujon et al., 2000). Nevertheless we cannot exclude a specific role of the expression of AtEPR1 expression in the germinating endosperm.

Specific activation of AtEPR1 and other genes in the endosperm may be one of the multiple events involved in facilitating the protrusion of the radicle during seed germination, or being a consequence of this protrusion. Their exact role in this process is still unknown. We are planning to use genetic ablation experiments, using an AtEPR1 promoter, to dissect the effective role of the genes expressed in the endosperm during seed imbibition.

Experimental procedures

Plant material

Arabidopsis thaliana (L.) Heyn., ecotype Wassilevskija (Ws), was used to generate a collection of T-DNA insertion mutants (Bechtold et al., 1993). Seeds derived from this collection were surface-sterilized and sown in Petri dishes (48 or 100 seeds per Petri dish), on solidified (0.7% agar) medium (Estelle and Somerville, 1987), or on two layers of water-moistened Whatman paper (No. 4) (Whatman, Maidstone, UK). Experiments testing the effect of ABA (Sigma, USA) or GAs (Sigma-Aldrich, Saint Quentin Fallavier, France) on the expression of the AtEPR1 gene were conducted with the same protocol, at the chosen concentration. Experiments using GA-biosynthesis mutants were conducted using the gat-3 mutant (Koorrieneef and Van der Veen, 1980) supplied by the Nottingham Arabidopsis Stock Center (NWS). Plants were directly placed in growth chambers kept at 60% moisture, under a light/dark regime of long days (16 h light, 25 µE m⁻² s⁻¹, 20°C/8°C dark, 15°C). Under these growth conditions approximately 100% germination efficiency for wild-type seeds was obtained.

Histochemical analysis

β-Glucuronidase (GUS) activities were assayed as described by Jefferson et al. (1987), and histochemically localized using an X-gluc staining solution consisting of 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl glucuronide (Duchefa, Haarlem, The
ammonium bromide according to the procedure of Doyle and Doyle (1990). Genomic DNA (2 μg) was digested with 15 units of restriction enzyme according to the recommendations of the suppliers (Gibco-BRL), electrophoresed through a 0.8% (w/v) agarose gel with 10% (v/v) loading buffer containing RNase A (1 μg·mL⁻¹), and transferred onto nylon filter (Hybond-N, Amersham, UK). The probes were labelled with [α³²P]dCTP using the random primer DNA-labelling kit (Pharmacia, Uppsala, Sweden). Hybridization was carried out at 65°C in 5 × SSPE, 0.5% (v/v) sarkosyl and 5 × Denhardt’s solution. Filters were washed in 2 × SSC, 0.5% sarkosyl, 0.2% sodium pyrophosphate for 10 min at room temperature, then in the same buffer for 20 min at 65°C, followed by a 0.2 × SSC, 0.5% sarkosyl, 0.2% sodium pyrophosphate wash at 85°C, and finally exposed to Kodak X-OMAT AR film.

Chimeric promoter constructs

The 5’ region (0.8 kb) of the AtEPR1 ORF was amplified by PCR, using a T-DNA specific primer and the 5’ primer (GATGAG-TGTGGCAGAGAAGC). The PCR product was blunt-end cloned into a pMECA vector (Thomson and Parrott, 1998). The plasmid carrying the promoter region was digested with the restriction enzymes HindIII and Smal, and the insert fused to a pBlues vector (Clontech, Palo Alto, USA) containing a promoterless β-glucuronidase reporter gene (GUS). The chimeric construct was introduced in Arabidopsis by in planta transformation according to Bechtold et al. (1993). Primary transformants were selected on solid agar medium supplemented with kanamycin (100 mg·L⁻¹) and the GUS expression pattern of the transformants was tested in their progeny.

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